Preferential Solvation of Bovine Serum Albumin in Aqueous Guanidine Hydrochloride

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SUMMARY

Bovine serum albumin, in aqueous guanidine hydrochloride, interacts preferentially with the solvent components, as was shown by techniques in which refractometry and light scattering and equilibrium dialysis were used. If constant salt molality after dialysis is taken as the reference state for zero binding, then 0.08 ± 0.03 and 0.18 ± 0.05 g of salt per g of protein is bound at 3 M and 6 M salt, respectively, with similar values at 4 and 5 M salt. These results indicate that $(\bar{v}_2)_{T,\mu_1,\mu_2}$ (obtained by measuring the difference in density between a protein solution and its dialysate) is 2 to 3% less than the value obtained at constant molality of salt.

In recent years, aqueous guanidine hydrochloride has been used extensively as a solvent for measuring the molecular weights of protein subunits (1–4). This solvent weakens noncovalent interactions between amino acid residues and thus eliminates association between subunits while causing the polypeptide chains to be unfolded.

In using such a three-component system, however, it is essential to account for interactions between the protein and the solvent components in order to obtain correct molecular weight values. For this purpose it is useful to determine the preferential binding of solvent components. To date, few studies of the preferential interaction between protein and the solvent components in aqueous guanidine hydrochloride have been reported. Kielley and Harrington (4) have found that, in 5 M guanidine HCl, approximately 0.05 g of salt are bound preferentially per g of protein in the case of ribonuclease and myosin. Schachman and Edelstein (3), on the other hand, reported the preferential binding of 0.14 to 0.2 g of H_2O per g of aldolase over a guanidine HCl concentration range of 3 to 7 M. We have undertaken a systematic study of the preferential binding of solvent components to proteins in three-component systems using light scattering and differential refractometry, and the results for the water-bovine serum albumin-guanidine HCl system are reported in this paper.

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THEORETICAL

The fluctuation theory of light scattering was examined first by Zernike more than 50 years ago (5). The three-component equation as expressed by Brinkman and Hermans (6), Kirkwood and Goldberg (7), and Stockmayer (8) may be written in the form (Component 1, water; Component 2, macromolecule; Component 3, salt)[1]

$$H\frac{c_2}{\Delta\tau} = \frac{1}{(1+D)M_2}\left\{1 + \left[\left(\frac{\partial\mu_2^{(e)}}{\partial c_2}\right)_{T,P,c_3} - \frac{M_2}{M_3}\frac{(\partial\mu_3/\partial c_2)^2_{T,P,c_2}}{(\partial\mu_3/\partial c_3)_{T,P,c_2}}\right]\frac{c_2}{RT}\right\} \quad (1)$$

$$1 + D = \left[1 - \frac{(\partial n/\partial c_3)_{T,P,c_2}}{(\partial n/\partial c_2)_{T,P,c_3}}\frac{(\partial\mu_2/\partial c_2)_{T,P,c_3}}{(\partial\mu_3/\partial c_3)_{T,P,c_2}}\right]^2$$

$$\mu_i = RT\log c_i + \mu_i^{(e)} + \mu_i^0\,(T,P)$$

$$H = \frac{32\pi^3 n^2(\partial n/\partial c_2)^2_{T,P,c_3}}{3(\lambda)^4 N}$$

where $\Delta\tau$ is the excess turbidity of solution over solvent, R is the gas constant, P is the pressure, T is the thermodynamic temperature, n is the refractive index of the solution, λ is the wave length of the light, N is Avogadro's number, $\mu_i^{(e)}$ is the excess chemical potential of component i, M_i is its molecular weight, and c_i is the concentration in grams per ml. In Equation 1, the term $(1 + D)$ represents the interaction between protein and solvent, *i.e.* the excess of binding of salt or water to the protein.[2] It has been shown (6–8, 10–20) that

[1] This is the notation of Stockmayer (8) and Scatchard (9); Kirkwood and Goldberg (7) denote the salt as Component 1; Vrij and Overbeek (10) denote protein as Component 1 and salt as Component 2.

[2] In the present context the term "binding" is taken in its broadest sense, namely as general thermodynamic interactions; the nature of the interactions is not specified, and no conclusion can be drawn on the formation of molecular complexes with

$$\frac{1}{\left(H\frac{c_2}{\Delta\tau}M_2\right)_{c_2\to 0}} = \left[1 + \frac{(\partial n/\partial c_3)_{T,P,c_2}}{(\partial n/\partial c_2)_{T,P,c_3}}\left(\frac{\partial c_3}{\partial c_2}\right)_{T,\mu_1,\mu_3}\right]^2 \quad (2)$$

where $(\partial c_3/\partial c_2)_{T,\mu_1,\mu_3}$ is a measure of the change in salt concentration (in grams per ml units) in the domain of the protein due to a change in protein concentration (in grams per ml units). In molal units, the similar expression is (10, 11)

$$\frac{1}{\left(M_2H\frac{c_2}{\Delta\tau}\right)_{c_2\to 0}} = \left[1 + \frac{(\partial n/\partial m_3)_{T,P,m_2}}{(\partial n/\partial m_2)_{T,P,m_3}}\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3}\right]^2 \quad (2a)$$

where m_i is the molal, or mole per 1000 g of water, concentration of component i. In Equation 2a, the parameter H contains refractive index increments measured with reference to molal concentrations, $(\partial n/\partial m_i)_{m_j}$, rather than grams per ml concentrations, $(\partial n/\partial c_i)_{c_j}$, as in Equations 1 and 2. Thus, in measuring preferential binding of solvent components by means of light scattering, it is essential to use consistent concentration units. If the binding term $[(\partial c_3/\partial c_2)_{T,\mu_1,\mu_3}$ or $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}]$ is positive, salt is bound preferentially, if it is negative, water is bound preferentially and the amount is given by

$$\left(\frac{\partial c_1}{\partial c_2}\right)_{T,\mu_1,\mu_3} = -\frac{c_1}{c_3}\left(\frac{\partial c_3}{\partial c_2}\right)_{T,\mu_1,\mu_3}$$
$$\left(\frac{\partial m_1}{\partial m_2}\right)_{T,\mu_1,\mu_3} = -\frac{m_1}{m_3}\left(\frac{\partial m_3}{\partial m_2}\right)_{T,\mu_1,\mu_3} \quad (3)$$

At the limit of zero protein concentration the preferential binding on a molal basis

$$\left(\frac{\partial g_3}{\partial g_2}\right)^0_{T,\mu_1,\mu_3}$$

may be related to that measured on a molar basis, $(\partial c_3/\partial c_2)^0_{T,\mu_1,\mu_3}$, by[3]

$$\left(\frac{\partial g_3}{\partial g_2}\right)^0_{T,\mu_1,\mu_3} = \frac{g_3}{c_3}\left[\left(\frac{\partial c_3}{\partial c_2}\right)^0_{T,\mu_1,\mu_3} - \left(\frac{\partial c_3}{\partial c_2}\right)^0_{T,P,g_3}\right]$$
$$\left(\frac{\partial c_2}{\partial c_2}\right)^0_{T,P,g_3} = -c_3\bar{v}_2^0 \quad (4)$$

where g_i is the concentration of component i in grams per g of H_2O and $\bar{v}_2$ is the partial specific volume of the macromolecule. The superscript 0 refers to infinite dilution of the macromolecule. Inspection of Equations 2 to 4 shows that the extent, and even the sign, of the preferential binding may be different, depending on whether one uses molar (or c_i) or molal (or g_i) concentration units.

The actual choice of units for expressing preferential interaction is arbitrary. The quantity

$$\left(\frac{\partial c_3}{\partial c_2}\right)_{T,\mu_1,\mu_3}$$

defines zero preferential binding as the state in which the salt concentration of a macromolecular solution and its dialyzate are of the same molarity; $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$, on the other hand, defines the zero preferential binding state as that in which the amount of salt per 1000 g of water is identical on the two sides of the dialysis membrane, at osmotic equilibrium. The value of the preferential binding depends on the concentration units used (18). In fact, it is possible to have preferential salt binding on a molal basis, but preferential water binding on a molar basis. The salt can be diluted on the molar basis by the addition of macromolecule while remaining at constant molality. This fact is expressed by Equation 4, in which the term $(\partial c_3/\partial c_2)^0_{T,P,g_3}$ represents the dilution (grams per ml basis) of salt by the addition of the macromolecule; this term is always negative. Inspection of this equation shows that, at low salt concentrations, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ approaches $(\partial c_3/\partial c_2)_{T,\mu_1,\mu_3}$, rendering the interpretation of preferential binding unambiguous.

An alternate way of determining preferential binding is by measurement of the difference between the values of the refractive index increment of the protein measured at constant chemical potential and at constant concentration of the salt (10, 11, 15–18). Thus

$$\left(\frac{\partial c_3}{\partial c_2}\right)_{T,\mu_1,\mu_2} = \frac{(\partial n/\partial c_2)_{T,\mu_1,\mu_3} - (\partial n/\partial c_2)_{T,P,c_3}}{(\partial n/\partial c_3)_{T,P,c_2}} \quad (5)$$

The refractive index increment of the protein at constant salt concentration is measured after dissolving the protein in a salt solution of the desired final concentration, using a salt solution of the same concentration as reference. In order to obtain $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$, a similar differential measurement is carried out between the protein solution and the salt solution with which it is in osmotic equilibrium; usually this can be closely approximated by measuring the difference in refractive indices between the protein solution and its dialyzate (10, 15–18).

While preferential binding may be measured by the deviation of the limiting value of the light-scattering envelope from the reciprocal of the true molecular weight, this approach is valid only if no true changes in molecular weight have occurred. Thus, a control experiment on molecular weight determination is desirable. Ooi (11) has shown (see also 10, 16–18) that combination of Equations 1 and 5 reduces the light scattering equation to a pseudo two-component form, with the intercept being independent of macromolecule-solvent interactions

$$H'\frac{c_2}{\Delta\tau} = \frac{1}{M_2}\left[1 + \left(\frac{\partial\mu_2^{(o)}}{\partial c_2}\right)_{T,\mu_1,\mu_3}\frac{c_2}{RT}\right] \quad (6)$$

where in H', $(\partial n/\partial c_2)^2_{T,\mu_1,\mu_3}$ replaces $(\partial n/\partial c_2)^2_{T,P,c_3}$ of Equation 1 and $\Delta\tau$ is the difference in turbidity between protein solution and dialyzate. The second, concentration-dependent term has been reduced to the two-component form, since measurements at osmotic equilibrium eliminate the cross-terms from the second virial coefficient as well (10, 11, 15–18). Thus, if the light scattering and refractive index increment measurements are carried out using initial salt solution and dialyzate as reference, respectively, it is possible to obtain two independent measurements of preferential solvation, as well as a control on the state of aggregation of the macromolecule.

EXPERIMENTAL PROCEDURE

Reagents—BSA[4] was purchased from Nutritional Biochemicals (Lots 8762 and 9385).[5] Since essentially the same results were

binding at specific sites. Preferential binding of a given component means stronger attraction to the protein of that component than of others (14).

[3] Equation 4 is equivalent to Equation 3.16 of Reference 18.

[4] The abbreviation used is: BSA, bovine serum albumin.

[5] Mention of companies or products is for the convenience of

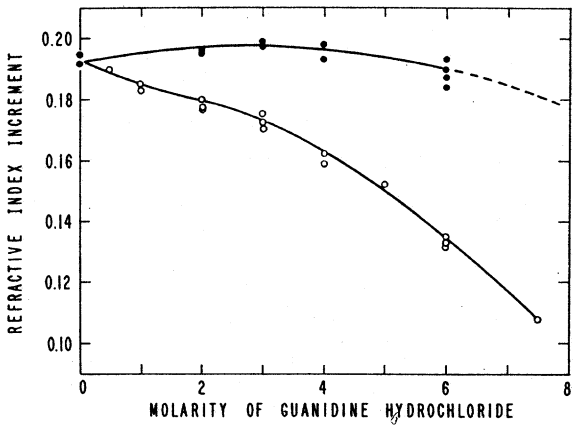

FIG. 1. Refractive index increment of BSA at various guanidine HCl concentrations (pH 5.2); 25°. *Upper curve*, constant guanidine HCl concentration, $(\partial n/\partial c_2)_{T,P,c_3}$. *Lower curve*, constant chemical potential of guanidine HCl and H_2O $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$.

obtained from the two lots, they will not be differentiated. Guanidine HCl was prepared from the carbonate (obtained from Eastman Organic Chemicals) by the method of Anson (21). Iodoacetamide, used to alkylate the sulfhydryl group of BSA, was a product of Aldrich Chemicals. The other reagents used were of analytical grade or the equivalent. Dialysis tubing was obtained from Union Carbide.

Concentration Determination—The protein concentration of a dialyzed BSA solution was determined by measuring the optical density of an aliquot suitably diluted with dialyzate. The absorbance of BSA at 278 mμ, A_{278}, at any given guanidine HCl concentration was determined on a solution prepared by diluting a deionized BSA solution of known concentration (dry weight at 107–109°) with concentrated guanidine HCl. Corrections were made for light scattering using the method described by Reddi (22): the amount of scattering was determined from the absorbance at 330 mμ. The absorptivity values are: 6.58 dl per g cm in 0 to 1.8 M guanidine HCl; 6.37 in 2.0 M guanidine HCl; 6.32 in 2.25 M guanidine HCl; 6.14 in 3.0 to 6.75 M guanidine HCl. The values given are the average of duplicate runs which agreed in all cases to better than 1%.

Refractive Increment—Differences in index of refraction between appropriate solutions, used in determining the refractive index increment, were measured with the Brice photoelectric differential refractometer at 25° at a wave length of 436 mμ.

The refractive index increment of guanidine HCl, $(\partial n/\partial c_3)_{T,P,c_2}$, at concentration c_3 was determined by comparison of solutions that differed slightly in molality (0.3 to 0.5 molal difference). The concentration differences on the gram per ml scale were determined by use of the equation of Kawahara and Tanford relating density to composition of aqueous guanidine HCl (23). The value of $(\partial n/\partial c_3)_{T,P,c_2}$ was constant over the concentration range of 2.5 to 7 M at a value of 0.178 ± 0.002 ml per g. At any given concentration of guanidine HCl, the value did not vary significantly when the concentration difference between the two solutions used for the measurement was varied.

Values of $(\partial n/\partial c_2)_{T,P,c_3}$ were determined by the method described by Katz (12) with slight modifications. A stock solution of guanidine HCl was delivered by pipette into a 25-ml volumetric flask which contained a small magnetic stirring bar of known volume (~0.15 ml). The weight of this added stock solution was also measured. Water was added to just below the line on the flask while the solution was being stirred. Care was taken to avoid getting liquid above the line. The flask was then placed in a 25.0 ± 0.005° water bath and filled to the calibration line. The solution was then restirred and again placed in the bath to insure proper filling. The contents were then transferred to an Erlenmeyer flask, which was then tightly stoppered. The companion solution, containing protein, was made with the same stock guanidine HCl solution, volumetric flask, and stirring bar. The added protein was introduced into the flask by pipetting a deionized solution of known concentration (dry weight, 107–109°). The refractive index difference between the protein solution and the solvent was corrected for the amount due to the small difference in guanidine HCl concentrations. Values of $(\partial n/\partial c_2)_{T,P,c_3}$ for BSA at pH 5.2 are shown by the *top curve* in Fig. 1. The least squares curve is given by the equation

$$(\partial n/\partial c_2)_{T,P,c_3} = 0.192 + 3.94 \times 10^{-3} B - 7.33 \times 10^{-4} B^2$$

where B is the molarity of guanidine HCl. This equation was used to compute $(\partial n/\partial c_2)_{T,P,c_3}$ at 5.0 and 7.5 M guanidine HCl in order to estimate the degree of binding of solvent components; all other calculations were based on the actual data. Assuming a possible error of 0.03 ml in filling the flask and a 0.05% error in the weight of guanidine HCl, the error in $(\partial n/\partial c_2)_{T,P,c_3}$ would be 2 to 3% at 3 M guanidine HCl, and 3–4% at 6 M guanidine HCl.

Values of $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$ (given in Fig. 1) were determined by comparison of a protein solution with its dialyzate. Solutions were dialyzed for periods of 3 to 7 days. In order to be sure that equilibrium had been reached in this time, a protein solution initially 3 M in guanidine HCl was dialyzed against several changes of 6 M guanidine HCl. The value of the refractive index

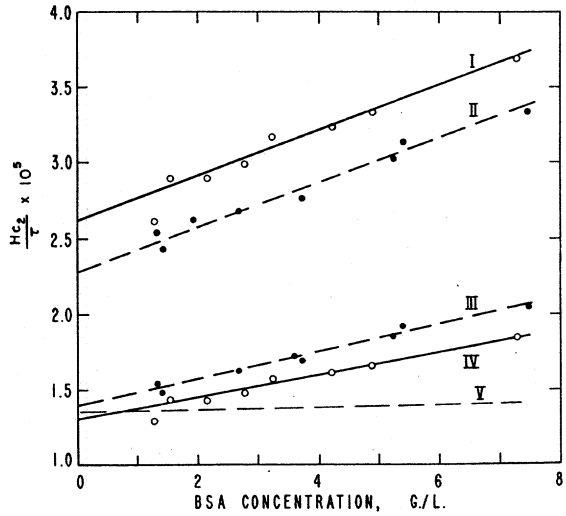

FIG. 2. Light scattering data on BSA. *Curves I* and *II*, 6 M guanidine HCl and 5 M guanidine HCl, respectively, (pH 5.2); 25°. Refractive index increment at constant guanidine HCl concentration used in calculations. *Curves III*, *IV*, and *V*, 5.0, 6.0, and 0.2 M guanidine HCl, respectively (pH 5.2); 25°. Refractive index increment at constant chemical potential of guanidine HCl and H_2O used in calculations.

the reader and does not constitute an endorsement by the United States Department of Agriculture.

TABLE I

Amount of preferential solvation

Concentration of guanidine HCl	$(\partial c_3/\partial c_2)_{T,\mu_1,\mu_3}$		$(\partial c_1/\partial c_2)_{T,\mu_1,\mu_3}$	$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$	$(\bar{v}_2)_{T,P,m_3} - (\bar{v}_2)_{T,\mu_1,\mu_3}$
	Refractometry	Light scattering			
M	*g/g BSA*		*g/g BSA*		*ml/g* × 10^2
3	−0.15 ± 0.02	—[a]	0.41	0.08 ± 0.03	1.4 ± 0.5
4	−0.20		0.37	0.10	1.6
5	−0.26	−0.26	0.35	0.14	1.9
6	−0.30 ± 0.03	−0.30	0.30	0.18 ± 0.05	2.1 ± 0.6
7.5	−(0.41 − 0.49)		0.26 − 0.31	0.09 ± 0.24	0.8 − 2.2

[a] BSA aggregates in 3 M guanidine HCl, even with iodoacetamide treatment; therefore, light scattering measurements would yield erroneous values for preferential solvation.

increment obtained was within 2% of that obtained after dialysis of a protein solution 6 M in guanidine HCl against 6 M guanidine HCl. Similar good agreement was found when a protein solution originally at 6 M guanidine HCl was dialyzed against 3 M guanidine HCl.

Light Scattering—Light scattering measurements were carried out at 25° using a slightly modified Dintzis technique (24–26). Since the angular dependence of the scattering of BSA in 6 M guanidine HCl was found to be negligible, the measurements were routinely made at 90° from the incident beam.

Preliminary light scattering experiments showed that unfolded BSA forms aggregates, probably because of sulfhydryl-disulfide interchange (27). Therefore, the sulfhydryl groups were blocked before significant interchange could take place, by dissolving the BSA (to 3%) in 5 M guanidine HCl-0.001 M EDTA-0.03 M iodoacetamide at pH 8.2 and 25°. The solution was maintained at pH 8.2 for 1 hour, adjusted to pH 5.2, and then dialyzed against the appropriate solvent for light scattering measurements. After such treatment the turbidity remained constant for 4 days. The light scattering results are shown in Fig. 2.

RESULTS AND DISCUSSION

Equations 2 to 5 are strictly valid only at infinite dilution of protein. They are valid, however, at finite concentration if $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$, $(\partial n/\partial c_2)_{T,P,c_3}$, and $(\partial n/\partial c_3)_{T,P,c_2}$ are independent of protein concentration. Our results for $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$ showed a possible concentration dependence only at 3 M guanidine HCl at which values of 0.170, 0.172, and 0.175 ml per g were obtained at 4.1, 7.4, and 6.9 g per ml of BSA, respectively. No trends were noted at other guanidine HCl concentrations. Also, Holtzer *et al.* (28) found a spread of only 0.0010 ml per g over the concentration range 4 to 10 g per liter for tropomyosin B in 5.0 M guanidine HCl-0.64 M KCl-0.06 M $K[PO_4]$, pH 6.1. Therefore, concentration dependence of the refractive index increment can be regarded as an unlikely source of error. The parameters $(\partial n/\partial c_2)_{T,P,c_3}$ and $(\partial n/\partial c_3)_{T,P,c_2}$ should show less dependence on protein concentration than $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$ because the latter includes contributions from redistribution of solvent components, while the others do not. The refractive index increment of the salt, $(\partial n/\partial c_3)_{T,P,c_2}$, was measured in the absence of protein with the assumption that addition of protein would have no effect on the value (10).

Values of $(\partial c_3/\partial c_2)_{T,\mu_1,\mu_3}$ and $(\partial c_1/\partial c_2)_{T,\mu_1,\mu_3}$ calculated from our data are given in Table I. The values at 7.5 M guanidine HCl are somewhat uncertain because $(\partial n/\partial c_2)_{T,P,c_3}$ was estimated from an extrapolation of the *top curve* of Fig. 1. All points on the curve are within experimental error of the average value, 0.195 ml per g, of all measured points. If $(\partial n/\partial c_2)_{T,P,c_3}$ at 7.5 M guanidine HCl is taken as 0.195 ml per g instead of 0.181 ml per g, $(\partial c_3/\partial c_2)_{T,\mu_1,\mu_3}$ would have a value of −0.49 g of guanidine HCl per g of BSA instead of −0.41.

The light scattering results in 0.20, 5.0, and 6.0 M guanidine HCl (pH 5.2) are shown in Fig. 2. The scatter of the data points about the *least squares lines I-IV* in Fig. 2 (5 and 6 M guanidine HCl) is representative of that found in 0.20 M guanidine HCl (*line V*). *Curves I* and *II* in Fig. 2 were calculated with use of $(\partial n/\partial c_2)_{T,P,c_3}$ in the light scattering equations and *Curves III*, *IV*, and *V* with the use of $(\partial n/\partial c_2)_{T,\mu_1,\mu_3}$. Use of the latter value should yield the true molecular weight.

Working at constant salt concentration, the apparent molecular weights found were 38,000 and 44,000 in 6 M and 5 M guanidine HCl, respectively. At constant chemical potential, the true values of M_2 were 73,000, 72,000, and 76,000 in 0.20, 5.0, and 6.0 guanidine HCl, respectively. The sample was not treated with iodoacetamide prior to use in 0.20 M guanidine HCl. Sedimentation patterns of the untreated BSA sample in 0.1 M KCl-0.001 M sodium acetate-0.001 M acetic acid showed ~5% of rapidly moving material in addition to the main component. This material is presumably dimers and higher polymers (29, 30) and its presence can account for our high values of the molecular weight. Furthermore, these values are in good agreement with values usually obtained by light scattering (14, 24, 31). Values of $(\partial c_3/\partial c_2)_{T,\mu_1,\mu_3}$ calculated from the light scattering data with use of Equation 2 are given in Table I.

Casassa and Eisenberg (18) have pointed out that the use of the term "binding" for the parameter $(\partial c_3/\partial c_2)_{T,\mu_1,\mu_3}$ may be ambiguous since it depends on an arbitrary choice of the reference state for zero binding. If equal salt molarity on both sides of the dialysis bag is chosen as the zero binding reference state, then the negative values of $(\partial c_3/\partial c_2)_{T,\mu_1,\mu_3}$ imply preferential binding of water with the amount given by the value of $(\partial c_1/\partial c_2)_{T,\mu_1,\mu_3}$; values of this parameter were calculated with the use of Equation 3 and are listed in Table I. It is seen that a given volume of BSA solution will contain about 0.3 g more water than its dialyzate for every gram of BSA dissolved.

It is of interest to evaluate $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, since this is the amount of salt preferentially bound on the commonly used molal scale; it represents the number of grams of salt per g of protein in a given weight of water, in excess of the dialyzate composition. Values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ have been calculated with the use of Equation 4 and are listed in Table I. A value of 0.734 ml per g for the partial specific volume $(\bar{v}_2)$ of BSA was used in the calcu-

lations (32). The amount of guanidine HCl preferentially bound corresponds to about 1 per 10 amino acid residues at 3 M guanidine HCl to 1 per 4 residues at 6 M guanidine HCl.[6] Since our methods measure the amount of salt or water bound in excess of the solvent composition, any binding of the salt and water in the same ratio as that found in the dialyzate would go undetected. Therefore, our values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ must be considered as minimal values of guanidine HCl actually bound, keeping in mind the ambiguities associated with the term "binding."

While the present study has been concerned with the optical techniques of light scattering and differential refractometry, multicomponent effects must be taken into account when measuring molecular weights by any method requiring thermodynamic measurements. Thus, in the case of sedimentation equilibrium and velocity, the problem has been discussed in recent years by Vrij (16), Jacob and Daune (33), Casassa and Eisenberg (18, 34), Cox and Schumaker (35), and Ifft and Vinograd (36). One should add, as pointed out by Scatchard (37), that the solution of the multicomponent problem in sedimentation equilibrium can be found already in a treatment by J. W. Gibbs in 1887 of the effect of a gravitational field on the chemical potential of a system. In sedimentation equilibrium, the counterpart of Equations 2 and 5 is

$$(\bar{v}_2)_{T,P,m} - (\bar{v}_2)_{T,\mu_1,\mu_3} = (\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}\,[1/\rho_s - (\bar{v}_3)_{T,P,m_2}] \qquad (7)$$

where $(\bar{v}_2)_{T,P,m_3}$ and $(\bar{v}_2)_{T,\mu_1,\mu}$ are the apparent partial specific volumes obtained by comparing the density of the macromolecule solution with that of the solvent and the dialyzate, respectively, ρ_s is the solution density, and $(\bar{v}_3)_{T,P,m_2}$ is the partial specific volume of the salt. Comparison of Equation 7 with Equations 2 and 5, and application of Equation 4, makes possible the calculation of $[(\bar{v}_2)_{T,P,m_3} - (\bar{v}_2)_{T,\mu_1\mu_3}]$ from light scattering or refractive index increment data, if the solution density is known.

In the case of BSA, it has been shown (38) that the value of $(\bar{v}_2)_{T,P,m_3}$ in 4 to 8 M urea differs very little from that found in water. Similar results have been reported for ovalbumin (39) and β-lactoglobulin (40). Assuming that the volume changes which occur when BSA is transferred from water to aqueous guanidine HCl are small, it is possible to calculate the difference between $(\bar{v}_2)_{T,P,m_3}$ in dilute salt and $(\bar{v}_2)_{T,\mu_1,\mu_3}$ in concentrated guanidine HCl. Values of this quantity have been calculated with use of Equation 7 and are given in Table I. The results show that $(\bar{v}_2)_{T,\mu_1,\mu_3}$ for BSA in concentrated guanidine HCl (pH 5.2) is 2 to 3% lower than the value of $(\bar{v}_2)_{T,P,m_3}$ in water. Similar small decreases for other proteins have been found by actual measurement of $(\bar{v}_2)_{T,\mu_1,\mu_3}$ (2, 4, 41, 42). Our results suggest further that $(\bar{v}_2)_{T,\mu_1,\mu_3}$ is only slightly sensitive to guanidine HCl concentration.

Kielley and Harrington (4) have found $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ of the order of 0.05 g of guanidine HCl per g of protein for ribonuclease and myosin in 5 M guanidine HCl by dry weight measurements on protein solution and dialyzate. Our values are somewhat greater; this might be due, however, to differences in methodology as well as in the different proteins used. Woods, Himmelfarb, and Harrington (43) have measured directly $(\bar{v}_2)_{T,P,m_3}$ and $(\bar{v}_2)_{T,\mu_1,\mu_3}$ for lobster myosin in 5 M guanidine HCl, with the result that the salt is bound preferentially to the protein to the extent of 0.14 g of guanidine HCl per g of protein; this value is in good agreement with that calculated in this paper for the preferential binding of guanidine hydrochloride to bovine serum albumin. Schachman and Edelstein (3) determined $(\bar{v}_2)_{T,\mu_1,\mu_3}$ of aldolase in aqueous guanidine HCl by sedimentation equilibrium measurements. They plotted $M[1 - (\bar{v}_2)_{T,\mu_1,\mu_3}\rho_s]$ versus ρ_s and extrapolated the line obtained to the density at which no redistribution of protein would occur. The value of $(\bar{v}_2)_{T,\mu_1,\mu_3}$ obtained from a knowledge of the buoyant density was 0.035 ml per g higher than the value in dilute salt. It was concluded that water was preferentially bound, *i.e.* $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ was negative, to the extent of 0.14 to 0.20 g per g of aldolase. Using the isopiestic method, Hade and Tanford (44) have examined a number of proteins in a 6 M guanidine HCl medium with the conclusion that the salt is bound preferentially in all the cases studied.

In order to test further the degree of accuracy with which preferential binding of solvent components can be measured, it would seem desirable to apply systematically several methods to a given protein. The refractometric and light scattering methods described in this paper should be useful in such studies, in particular when they are complemented by other techniques, such as small angle X-ray scattering (45, 46) and sedimentation equilibrium and velocity.

[6] Preferential guanidine HCl binding of similar magnitude occurs in the four-component system, H_2O-guanidine HCl-bovine α_{s1}-casein B-potassium phosphate (pH 7). Values of $(\partial n/\partial c_2)_{T,\mu,\mu_{13},\mu_5}$ were found to be 0.173 ml per g and 0.128 ml per g at 3 and 6 M guanidine HCl, respectively; the potassium phosphate concentration was 0.1 M. Values of $(\partial n/\partial c_2)_{T,P,c_3,c_5}$ (Component 5 is potassium phosphate) were 0.192 ml per g and 0.195 ml per g at 3 and 6 M guanidine HCl, respectively. Katz has presented evidence that preferential binding of a fourth component to BSA should be negligible in the presence of a denaturant of much greater concentration than the fourth component. With this assumption, the value of the refractive index increments of α_{s_3}-casein B indicate preferential guanidine HCl binding (grams per g of H_2O basis) of 0.13 ± 0.04 g per g of protein and 0.07 ± 0.005 g per g of protein at 3 and 6 M guanidine HCl, respectively.

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